

# The *Saccharomyces cerevisiae* Suppressor of Choline Sensitivity (*SCS2*) Gene Is a Multicopy Suppressor of *mec1* Telomeric Silencing Defects

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## ABSTRACT

Mec1p is a cell cycle checkpoint protein related to the ATM protein kinase family. Certain *mec1* mutations or overexpression of Mec1p lead to shortened telomeres and loss of telomeric silencing. We conducted a multicopy suppressor screen for genes that suppress the loss of silencing in strains overexpressing Mec1p. We identified *SCS2* (suppressor of choline sensitivity), a gene previously isolated as a suppressor of defects in inositol synthesis. Deletion of *SCS2* resulted in decreased telomeric silencing, and the *scs2* mutation increased the rate of cellular senescence observed for *mec1-21 tel1* double mutant cells. Genetic analysis revealed that *Scs2p* probably acts through a different telomeric silencing pathway from that affected by Mec1p.

**I**N the yeast *Saccharomyces cerevisiae*, chromosomes terminate in a simple repetitive sequence [poly(G<sub>1-3</sub>T)] that is ~350–500 bp in length (GREIDER 1996). The telomeric repeats are packaged into a non-nucleosomal type of chromatin (WRIGHT *et al.* 1992). Telomere chromatin structure prevents the transcription of reporter genes at the telomere, a phenomenon called telomere position effect (TPE) or telomeric silencing (GOTTSCHLING *et al.* 1990).

Telomeric silencing requires a number of proteins that bind at the telomere. For example, the Rap1p (repressor and activator protein) binds directly to telomeric DNA (GILSON *et al.* 1993). Rap1p then recruits the Sir3 and Sir4 (silent information regulator) proteins (MORETTI *et al.* 1994; HECHT *et al.* 1995, 1996). The Rif1 protein (Rap1p-interacting factor; HARDY *et al.* 1992) competes with Sir3p for binding to Rap1p and acts as a negative regulator of telomeric silencing (KYRION *et al.* 1993; MORETTI *et al.* 1994). Additional proteins involved in regulating telomeric silencing include the H3 and H4 histones, proteins involved in regulating post-translation modifications of histones, and the DNA end-binding Ku proteins (reviewed by LUSTIG 1998). Many of these proteins also regulate silencing of the silent mating-type loci (APARICIO *et al.* 1991).

The Mec1p (mitotic entry checkpoint) directs the cellular response to DNA damage and S-phase arrest (ALLEN *et al.* 1994; WEINERT *et al.* 1994) and regulates telomere length (RITCHIE *et al.* 1999). The *mec1-21* allele or overexpression of the wild-type *MEC1* results in loss of telomeric silencing (CRAVEN and PETES 2000); some mutant

alleles of *MEC1* result in shortened telomeres without loss of telomeric silencing (LONGHESE *et al.* 2000). The *mec1-21* silencing defect can be suppressed by a mutation in the *SML1* (suppressor of *mec1* lethality) gene (CRAVEN and PETES 2000); mutations in *SML1* result in elevated nucleotide pools (ZHAO *et al.* 1998). Mec1p directs a signaling cascade that includes the Dun1p kinase (ZHOU and ELLEDGE 1993), and *dun1* cells also exhibit shortened telomeres and decreased telomeric silencing (CRAVEN and PETES 2000; LONGHESE *et al.* 2000).

Strains with mutations in both *MEC1* and the related *TEL1* gene (LUSTIG and PETES 1986; GREENWELL *et al.* 1995) have very short telomeres and undergo cellular senescence (RITCHIE *et al.* 1999). Following ~50 generations of attenuated growth, “survivor” colonies appear by a recombination-dependent mechanism (RITCHIE *et al.* 1999). In summary, the related Mec1p and Tellp are required for telomere length regulation; Mec1p, but not Tellp, also has a role in telomeric silencing. Similar observations have also been made in *Schizosaccharomyces pombe*. Strains with mutations in both *rad3<sup>+</sup>* (the gene equivalent to *MEC1*) and *tel1<sup>+</sup>* undergo complete loss of telomeres (NAITO *et al.* 1998), and strains with single mutations in the *rad3<sup>+</sup>* gene lose telomeric silencing (DAHLEN *et al.* 1998; MATSUURA *et al.* 1999). Thus, the functions of *MEC1* at the telomere are widely conserved through evolution.

Since mutations within the kinase domain of Mec1p affect telomere length (MALLORY and PETES 2000), it is likely that the effects of the *mec1* mutation on telomere length and telomeric silencing are a consequence of lack of phosphorylation of downstream targets. Although Mec1p-dependent phosphorylation of a number of proteins involved in the repair of DNA damage has been observed (reviewed by LOWNDES and MURGUIA 2000),

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the targets of Mec1p relevant to its effects at the telomere are unknown. One way to search for downstream targets of a protein is to screen for genes that, when overexpressed, suppress mutant phenotypes. Such multicopy suppressors may be downstream targets of the signaling protein, genes that activate competing pathways, or genes that inactivate inhibitory pathways (GUTHRIE and FINK 1991). Below, we describe a screen for genes that, when overexpressed, suppress the telomeric silencing defect caused by overexpression of *MEC1*.

## MATERIALS AND METHODS

**Yeast strains:** All strains were isogenic with W303a (*leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535*; THOMAS and ROTHSTEIN 1989), except for alterations introduced by transformation. The genotypes of strains used in our study are shown in Table 1. The names and sequences of oligonucleotides used for strain constructions or strain diagnosis are given in Table 2.

Most W303a-derived strains contain the *rad5-535* mutation (FAN *et al.* 1996). We isolated *RAD5* derivatives of such strains by crossing them to the isogenic *RAD5* strain W1588-4C (from R. Rothstein). The presence of the *rad5-535* mutation was scored by PCR amplification of genomic DNA with the primers RAD5-L and RAD5-R and treatment of the resulting DNA fragment with the *MnII* restriction enzyme. The *rad5-535* substitution introduces an *MnII* site into the *RAD5* coding sequence. In direct comparisons of *RAD5* and *rad5-535* strains, we found no differences in telomeric silencing.

A number of strains with deletions were constructed using the PCR method described by WACH *et al.* (1994). The *SCS2* (suppressor of dholine sensitivity) gene was replaced by *HIS3* using a PCR fragment (primers, *SCS2-KOF* and *SCS2-KOR*; template, pRS303) in a one-step transplacement. The *INO1* gene was replaced by *HIS3* using a similar approach (primers, *INO1-KOF* and *INO1-KOR*; template, pRS303). In three strains, genes were replaced with the *kanMX* gene (WACH *et al.* 1994), which confers resistance to geneticin, by the same procedure. These genes and the primers used to generate the PCR fragment for the one-step transplacements were: *RAD9* (*RAD9-KOF* and *RAD9-KOR*), *TEL1* (*TEL1-KOF* and *TEL1-KOR*), and *YBL091C-A* (*YBL-KOF* and *YBL-KOR*); the template for the PCR reactions was pFA6-*kanMX* (WACH *et al.* 1994).

We also used PCR methods to epitope-tag Sir3p and Scs2p. The primers *SIR3-F* and *SIR3-R* for Sir3p and *SCS2-F* and *SCS2-R* for Scs2p (sequences in Table 2) were used to amplify the plasmid pFA6a-3HA-*kanMX6* (LONGTINE *et al.* 1998). The resulting DNA fragments were used to transform W1588-4c to geneticin resistance. In one of the resulting strains (RCY309), the Scs2p contains two hemagglutinin (HA) epitopes inserted immediately upstream of the termination codon and there is an insertion of the *kanMX* cassette downstream of *SCS2*. In the second strain (RCY310), the Sir3p has the same 2XHA tag immediately upstream of the termination codon with the same *kanMX* insertion downstream of *SIR3*.

We assayed telomeric silencing using a construction in which the *URA3* gene was inserted near the end of chromosome XV<sub>L</sub> (GOTTSCHLING *et al.* 1990; CRAVEN and PETES 2000). This construction was introduced into various genetic backgrounds by crosses. The resulting diploids (haploid strains shown in parentheses) were: RCY165 (RCY109-15d × W303aU-fr), RCY207 (RCY201 × W303a), RCY211 (RCY207-3a × RCY109-1c), RCY243 (RCY242 × RCY109-25c), RCY269 (RCY268 ×

RCY243-7a), RCY278 (RCY273 × RCY243-7d), RCY280 (RCY28 × RCY243-1a), RCY282 (RCY269-7c × LPV253), RCY300 (Y286 × RCY269-4a), RCY305 (RCY269-4a × RCY278-1a), RCY307 (MD89 × RCY300-6a), and RCY346 (RCY106-1d × RCY211-2b).

**Multicopy suppressor screen:** The strain RCY138, containing a TEL-XV<sub>L</sub>-*URA3* telomere and the *MEC1*-containing plasmid pRC5, is sensitive to 5-fluoro-orotate (5-FOA) because overexpression of Mec1p results in loss of telomeric silencing (CRAVEN and PETES 2000). We transformed this strain with a YEp13-borne genomic library (DEMARINI *et al.* 1997), looking for transformants that had restored silencing. Transformants were selected on plates lacking both histidine (to maintain selection of pRC5) and leucine. Following 3 days of growth, colonies were replicated to plates lacking histidine and leucine but containing 1 mg/ml 5-FOA. Of ~12,000 His<sup>+</sup> Leu<sup>+</sup> transformants examined, only 40 were resistant to 5-FOA. Further analysis showed that only 6 of these transformants suppressed the silencing defect caused by Mec1p overexpression in a plasmid-dependent manner. Plasmids were rescued from each of the 6 transformants into *Escherichia coli*; these plasmids were called pMOS2 (*Mec1p*-overexpression suppression 2), pMOS7, pMOS13, pMOS21, pMOS24, and pMOS35.

**Plasmids:** The plasmid pMOS2 (described above) had two open reading frames. The open reading frame (ORF) representing the *SCS2* gene was subcloned as a 1.6-kb *HindIII*-*BglII* fragment into the *Bam*HI and *Hind*III sites of the *LEU2*-containing vector YEplac181 (GETZ and SUGINO 1988), resulting in the plasmid pRC12. The high-copy-number *LEU2*-containing pRC11 plasmid contains an insertion of the *RNR1* gene (CRAVEN and PETES 2000). The plasmid pRC5 is a high-copy-number *HIS3*-containing plasmid with *MEC1* (CRAVEN and PETES 2000), and pRC4 (identical to the previously described pRS4; CRAVEN and PETES 2000) is a *CEN*- and *HIS3*-containing plasmid with the *MEC1* gene. The plasmid pRS423 (CHRISTIANSON *et al.* 1992) is a high-copy-number *HIS3*-containing vector that was used as a control in some experiments. The *SIR3* overexpression plasmid pLP304 contains a 4.5-kb fragment of *SIR3* inserted into the *LEU2*-marked 2- $\mu$ m vector YEpl351 (STONE and PILLUS 1996). The plasmid pJH318 (HIRSCH and HENRY 1986) contains the *INO1* gene inserted into YEpl351. The plasmid pBAD45 (provided by S. Elledge) has an insertion of *MEC1* on a *CEN-URA3*-containing vector.

**Genetic methods, assays for silencing, and measuring sensitivity to DNA damaging agents:** Standard methods were used for transformation, media preparation, and tetrad analysis (GUTHRIE and FINK 1991). Because some mutant phenotypes associated with *mec1* or *tel1* mutations exhibit a substantial phenotype lag, strains with these mutations were subcloned for ~100 cell generations before monitoring any phenotypes.

Telomeric silencing assays were performed as described previously (CRAVEN and PETES 2000). Strains were grown overnight in rich growth medium (for plasmid-free strains) or appropriate synthetic media lacking specific amino acids. Cells were suspended in water and diluted 1:5 in serial increments, and 5  $\mu$ l of the diluted suspensions was spotted on rich growth medium (YPD) or plates containing 1 mg/ml 5-FOA. For some strains, synthetic media lacking histidine and/or leucine were used to force retention of *HIS3*- and/or *LEU2*-containing plasmids. To test silencing of the silent mating-type locus, we used strains that contained an insertion of *TRP1* integrated at the *HML* locus. In wild-type strains, silencing results in a Trp<sup>-</sup> phenotype (NISLOW *et al.* 1997).

Sensitivity to inhibition of growth by hydroxyurea was examined using medium containing 50–200 mM hydroxyurea. The concentration of the DNA-damaging agent methyl methane-sulfonate in the medium was 0.05%. To assay the ability of strains to grow in the absence of inositol or choline, we used

TABLE 1  
Haploid strains

Name	Genotype	Construction or reference
W303a	Wild type <sup>a</sup>	THOMAS and ROTHSTEIN (1989)
W303α	α	THOMAS and ROTHSTEIN (1989)
W1588-4C	<i>RAD5</i>	R. Rothstein
LPY253	<i>hml::TRP1</i>	STONE and PILLUS (1996)
MD89	<i>RAD5 rad9Δ::kanMX</i>	Transformation of W1588-4C with PCR fragment <sup>b</sup>
Y286	α <i>dun1-Δ100::HIS3</i>	ZHOU and ELLEDGE (1993)
W303aU-fr	<i>hdf1Δ::ura3</i>	PORTER <i>et al.</i> (1996)
Y602	<i>mec1Δ::HIS3</i> + pBAD45	DESANY <i>et al.</i> (1998)
RCY28	α <i>rif1Δ::kanMX</i>	CRAVEN and PETES (1999)
RCY106-1d	<i>rif1Δ::kanMX TELXV<sub>L</sub>::URA3</i>	CRAVEN and PETES (2000)
RCY109-2b	<i>TELXV<sub>L</sub>::URA3</i>	CRAVEN and PETES (2000)
RCY109-1c	<i>mec1-21 TELXV<sub>L</sub>::URA3</i>	CRAVEN and PETES (2000)
RCY109-15d	α <i>sml1Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	CRAVEN and PETES (2000)
RCY109-25c	α <i>mec1-21 TELXV<sub>L</sub>::URA3</i>	CRAVEN and PETES (2000)
RCY124-2a	<i>rap-17 hml::TRP1</i>	CRAVEN and PETES (2000)
RCY138	<i>TELXV<sub>L</sub>::URA3</i>	CRAVEN and PETES (2000)
RCY144-4a	<i>dun1-Δ100::HIS3 TELXV<sub>L</sub>::URA3</i>	CRAVEN and PETES (2000)
RCY165-1c	<i>hdf1Δ::ura3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY165
RCY201	<i>RAD5 ino1Δ::HIS3</i>	Transformation of W1588-4C with PCR fragment
RCY207-3a	α <i>RAD5 ino1Δ::HIS3</i>	Spore derivative of RCY207
RCY211-2b	α <i>ino1Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY211
RCY242	<i>RAD5 scs2Δ::HIS3</i>	Transformation of W1588-4C with PCR fragment <sup>b</sup>
RCY243-1a	<i>scs2Δ::HIS3 RAD5 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY243
RCY243-7a	α <i>RAD5 mec1-21 scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY243
RCY243-7d	α <i>RAD5 mec1-21 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY243
RCY268	<i>RAD5 YBL091C-AΔ::kanMX</i>	Transformation of W1588-4C with PCR fragment <sup>b</sup>
RCY269-1b	<i>RAD5 mec1-21 scs2Δ::HIS3 YBL091C-AΔ::kanMX TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY269
RCY269-2b	<i>RAD5 YBL091C-AΔ::kanMX TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY269
RCY269-3d	<i>RAD5 mec1-21 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY269
RCY269-4a	<i>RAD5 scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY269
RCY269-6a	<i>RAD5 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY269
RCY269-7c	α <i>RAD5 mec1-21 scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY269
RCY269-13c	<i>RAD5 scs2Δ::HIS3 YBL091C-AΔ::kanMX TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY269
RCY273	<i>RAD5 tel1Δ::kanMX</i>	Transformation of W1588-4C with PCR fragment <sup>b</sup>
RCY278-1a	α <i>RAD5 mec1-21 tel1Δ::kanMX TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY278
RCY280-1b	α <i>RAD5 rif1Δ::kanMX TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY280
RCY280-3b	<i>RAD5 rif1Δ::kanMX scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY280
RCY280-4b	α <i>RAD5 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY280
RCY280-6b	<i>RAD5 scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY280
RCY282-2a	<i>hml::TRP1</i>	Spore derivative of RCY282
RCY282-7c	α <i>hml::TRP1</i>	Spore derivative of RCY282
RCY282-11c	<i>scs2Δ::HIS3 hml::TRP1</i>	Spore derivative of RCY282
RCY282-13d	α <i>scs2Δ::HIS3 hml::TRP1</i>	Spore derivative of RCY282
RCY300-6a	α <i>RAD5 scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY300
RCY305-7a	<i>RAD5 tel1Δ::kanMX TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY305
RCY305-7b	<i>RAD5 mec1-21 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY305
RCY305-7c	<i>RAD5 tel1Δ::kanMX scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY305
RCY305-7d	<i>RAD5 mec1-21 scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY305
RCY305-9a	<i>RAD5 scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY305
RCY305-9b	<i>RAD5 mec1-21 tel1Δ::kanMX scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY305
RCY305-9c	<i>RAD5 mec1-21 tel1Δ::kanMX TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY305
RCY305-9d	<i>RAD5 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY305
RXY307-2c	<i>RAD5 rad9Δ::kanMX scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY307
RCY307-3c	<i>RAD5 scs2Δ::HIS3 TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY307
RCY307-4a	<i>RAD5 rad9Δ::kanMX TELXV<sub>L</sub>::URA3</i>	Spore derivative of RCY307
RCY309	<i>RAD5 SCS2-2HA/kanMX</i>	Transformation of W1588-4C with PCR fragment <sup>b</sup>
RCY310	<i>RAD5 SIR3-2HA/kanMX</i>	Transformation of W1588-4C with PCR fragment <sup>b</sup>

<sup>a</sup> The genotype of W303a is a *leu2-3,112 his3-11 ura3-1 ade2-1 trp1-1 can1-100 rad5-535*. All strains in this table are isogenic with W303a except for the changes indicated in the genotype column.

<sup>b</sup> The PCR primer sequences are in Table 2, and the PCR templates are described in MATERIALS AND METHODS.



TABLE 2

Name and sequence of oligonucleotides used in strain constructions

Name	DNA sequence (5' to 3')
<i>INO1</i> -KOF	ATTGGAGCTTTTCGTCACCTTTTTTTGGCTTGTCTGTTGTGCGGTTCCCTAGATTGTAAGTGCAGGTCGAC
<i>INO1</i> -KOR	TGTTTTTTTATAGGTAGGCGGAAAAAGAAAAAGAGAGTCGTTGAAATGAGCTGTGCGGTATTTTCACACCG
<i>RAD5</i> -L	GCAGCAGGACCATGTAAACG
<i>RAD5</i> -R	AACTCGTTACTCCACTGCG
<i>RAD9</i> -KOF	AGAAACGCCATAGAAAAGAGCATAGTGAGAAAATCTTCAACATCAGGGCTCGTACGCTGCAGGTCGAC
<i>RAD9</i> -KOR	TATTTAATCGTCCCTTTCTATCAATTATGAGTTTATATATTTTTATAATTATCGATGAATTCGAGCTCG
<i>SCS2</i> -KOF	TGTGTTAATAGTGTAGCAGAAGGGTATTCTACAATCTCCGGAACCTAAGTGATTGTAAGTGCAGGTCGAC
<i>SCS2</i> -KOR	ATATAAATATATATTTAGAATACAGCTATATCCTCAATCTCCCTACTGTGCGGTATTTTCACACCG
<i>TEL1</i> -KOF	GGAAATTCGAAAAAAAAGCCTTCAAAGAAAAGGGAAATCAGTGTAACATAGACGATGGAGCGTACGCT
	GCAGGTCGAC
<i>TEL1</i> -KOR	CGTATTTCTATAAAACAAAAAAGAAGTATAAAGCATCTGCATACCAATTAATCGATGAATTCGACCTCG
<i>YBL</i> -KOF	TTGCTACCTTTTTTGGTGCGATGCAACAGGTTACTAATATGTAATACTTCACGTACGCTGCAGGTCGAC
<i>YBL</i> -KOR	AAGCAATGCTCTGGAACCTCAGACTTTGCCGTTACCTGCTGATGGCCGCTCCATCGATGAATTCGAGCTCG
<i>SIR3</i> -F	ATAAATTACGCCTTTTCGATGGATGAAGAATTCAAAAATATGGACTGCATTTATCCCTATGACGTCCCG
<i>SIR3</i> -R	GAATACAGAGACTGCATGTGTACATAGGCATATCTATGGCGGAAGTGAATTCGAGCTCGTTTAAAC
<i>SCS2</i> -F	TATTCATATTGGTTGCACTCCTTATCTTGGTTTTAGGATGGTTCTACAGATATCCCTATGACGTCCCG
<i>SCS2</i> -R	CACATATATAAATATATTTAGAATACAGCTATATCCTCAATCTCCCTAGAATTCGAGCTCGTTTAAAC

vitamin-defined synthetic medium as defined by GRIAC *et al.* (1996).

**Chromatin immunoprecipitation:** Yeast strains (RCY309 with HA-tagged Scs2p and RCY310 with HA-tagged Sir3p) were grown in rich growth medium to an OD<sub>600</sub> of 1–1.5. The cells were treated with 1% formaldehyde for 2 hr. Crosslinking was stopped with 1 M glycine, and cell extracts were prepared as described by MELUH and KOSHLAND (1997). Immunoprecipitation, deproteinization, and PCR were performed as described by STRAHL-BOLSINGER *et al.* (1997). The antibody used for both immunoprecipitations and Western analysis (MALLORY and PETES 2000) was HA.11 (Babco, Richmond, CA). Telomeric sequences were detected by PCR using primers homologous to the V<sub>L</sub> telomere (MILLS *et al.* 1999).

## RESULTS

**Identification of *SCS2* as a multicopy suppressor of *mec1* TPE defects:** Cells that overexpress *MEC1* lack the ability to silence a telomeric *URA3* gene and, therefore, fail to grow on plates containing 5-FOA (CRAVEN and PETES 2000). We conducted a screen for genes that, when overexpressed, suppress the *MEC1* overexpression-silencing defect (details in MATERIALS AND METHODS). In a screen of ~12,000 transformants, we identified six different pMOS plasmids that were capable of suppressing the silencing defect. The identities of yeast genomic DNA within five of these plasmids were determined by DNA sequencing each junction of the insertion and by comparing the sequences with the *Saccharomyces* Genome Database. The chromosomal coordinates for each insertion were as follows (Roman numerals indicating the chromosome): pMOS2 (V, 26753–30712), pMOS7 (XV, 291737–295525), pMOS13 (VII, 77187–83915), pMOS21 (IX, 404000–410409), and pMOS24 (V, 509064–513891). The strongest suppression of the silencing defect was observed for the pMOS2

plasmid, and our subsequent analysis was restricted to this plasmid.

The plasmid pMOS2 contained two open reading frames. We constructed a plasmid (pRC12) that had one of these ORFs (YER120W, *SCS2*) and showed that this plasmid suppressed the silencing defect caused by *MEC1* overexpression (Figure 1a). *SCS2* is a protein of unknown function that has genetic interactions with proteins involved in inositol/lipid biosynthesis (KAGIWADA *et al.* 1998).

In addition to suppressing the telomeric silencing defect resulting from *MEC1* overexpression, *SCS2* overexpression suppressed the telomeric silencing defect of *mec1-21* and *dun1-Δ100* strains. While the *mec1-21* and *dun1Δ* strains RCY109-1c and RCY144-4a harboring a control vector grew poorly on medium with 5-FOA, indicating a silencing defect (Figure 1b), the same strains silenced at wild-type levels upon *SCS2* overexpression (Figure 1b). In contrast, *SCS2* overexpression did not suppress the telomeric silencing defect of cells lacking the *yKU70/HDF1* gene (Figure 1b), which encodes a DNA end-binding protein required for silencing (BOULTON and JACKSON 1998). Thus, the restoration of silencing by *SCS2* overexpression is not generalizable to all telomeric silencing mutants.

Strains with *mec1* or *dun1* mutations fail to form colonies in media containing hydroxyurea (HU, an inhibitor of ribonucleotide reductase; ZHOU and ELLEDGE 1993; ALLEN *et al.* 1994) and null mutants of *MEC1* are inviable (ZHAO *et al.* 1998). The inviability of *mec1* null mutants, but not the inability to form colonies on HU-containing media, is suppressed by overexpression of *RNR1* (DESANY *et al.* 1998), a gene encoding one of the subunits of ribonucleotide reductase. Overexpression of *SCS2* did not rescue the ability of *mec1-21* (Figure 2a) or *dun1*

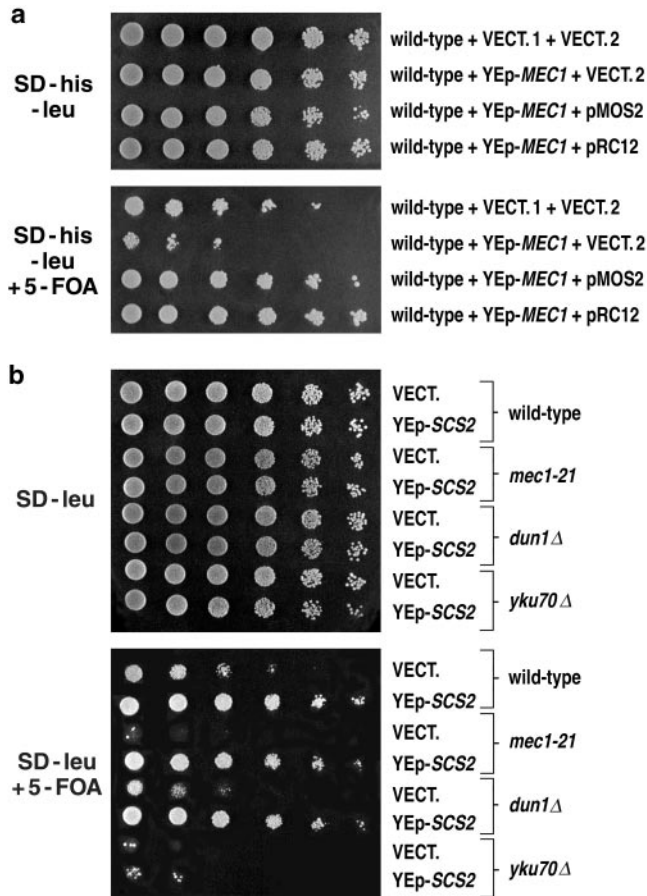


FIGURE 1.—*SCS2* suppresses telomeric silencing defects. All strains (derived from RCY138 by transformation with various plasmids) contained an insertion of *URA3* near the left telomere of chromosome XV (*TELXV<sub>L</sub>::URA3*); in RCY138, expression of *URA3* is turned off by telomeric silencing, resulting in a high frequency of 5-FOA<sup>R</sup> cells. (a) Suppression of the telomeric silencing defect caused by overexpression of Mec1p. Cells with various *HIS3*- and *LEU2*-containing plasmids were diluted in water and spotted on plates lacking leucine and histidine (top) or similar plates containing 5-FOA to assay telomeric silencing (bottom). Wild-type RCY138 cells containing two control plasmids (VECT.1, pRS423; VECT.2, YEp*lacI81*) silenced normally, while the same cells harboring the *MEC1* overexpression plasmid (YEp-*MEC1*, pRC5) silenced poorly. This loss of silencing was suppressed by the pMOS2 plasmid identified by screening, and by a subclone of pMOS2 (pRC12) containing only the *SCS2* gene. (b) *SCS2* suppresses the *mec1-21* and *dun1* silencing defects. Wild-type (RCY109-2b), *mec1-21* (RCY109-1c), *dun1*Δ (RCY144-4a), and *yku70* (RCY165-1c) cells were transformed with a control plasmid YEp*lacI81* (VECT.) or the *SCS2* overexpression plasmid pRC12 (YEp-*SCS2*).

(data not shown) strains to form colonies on HU-containing media. *SCS2* overexpression also did not suppress the essential function of *MEC1*. A strain (Y602) with a *mec1*Δ deletion and the plasmid pBAD45 (*CEN*-containing plasmid with *URA3* and *MEC1*) was transformed with a high-copy-number control plasmid (YEp*lacI81*), a high-copy-number *SCS2*-containing plasmid (pRC12), or a high-copy-number *RNR1*-containing plas-

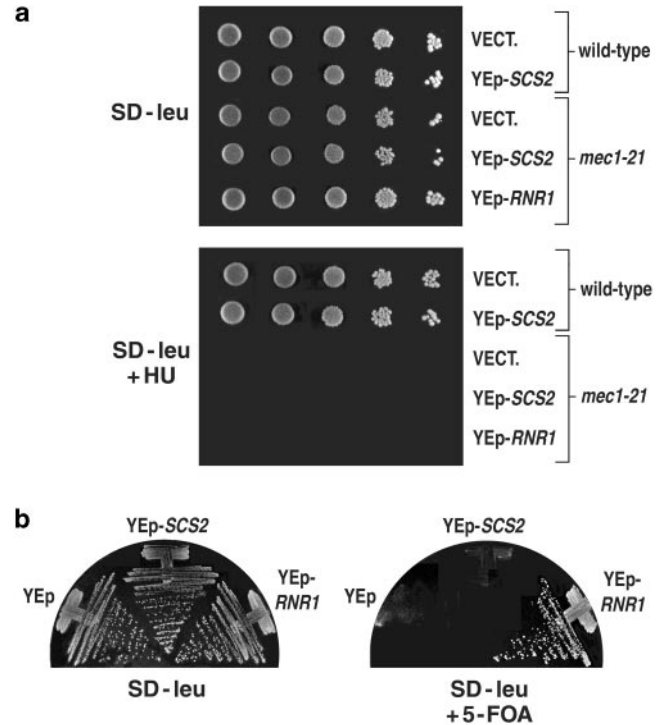


FIGURE 2.—*SCS2* does not suppress the role of Mec1p in the S-phase checkpoint response or the essential function of Mec1p. (a) Wild-type (RCY109-2b) or *mec1-21* (RCY109-1c) cells were transformed with a control plasmid YEp*lacI81* (VECT.), the *SCS2* overexpression plasmid pRC12 (YEp-*SCS2*), or the *RNR1* overexpression plasmid pRC11 (YEp-*RNR1*). Cells were plated onto media lacking leucine (top), or on plates lacking leucine and containing 50 mM hydroxyurea (HU, bottom). (b) A *mec1*Δ strain (Y602) harboring a *MEC1*-*CEN*-*URA3* plasmid (pBAD45) was transformed with a control plasmid (VECT., YEp*lacI81*), YEp-*SCS2* (pRC12), or YEp-*RNR1* (pRC11). The strain with the YEp-*RNR1* plasmid formed colonies on the 5-FOA plate because *RNR1* can suppress the essential function of Mec1p, allowing the strain to lose the *MEC1*-*CEN*-*URA3* plasmid. The lack of growth on 5-FOA plates of the strain with YEp-*SCS2* plasmid indicates that *SCS2* cannot suppress the essential function of Mec1p.

mid (pRC11). The ability of these strains to lose the *MEC1*-containing plasmid was monitored using medium containing 5-FOA. Only the strain with plasmid pRC11 was able to lose the *MEC1*-containing plasmid (Figure 2b).

**The *scs2*Δ mutation causes loss of telomeric silencing:** Deletion of the *SCS2* open reading frame caused a loss of telomeric silencing, similar to that observed for the *mec1-21* (Figure 3) and *dun1*Δ (data not shown) mutants. We measured telomeric silencing in five independent cultures of isogenic wild-type, *scs2*, *mec1-21*, and *scs2 mec1-21* strains. The percentages of cells in each culture that were 5-FOA<sup>R</sup> (range of values shown in parentheses) were : 10% (4.4–14%) for wild type, 0.8% (0.5–1%) for *scs2*, 1.9% (1.5–2.7%) for *mec1-21*, and 0.06% (0.03–0.1%) for *scs2 mec1-21*. Telomere length was unaffected by deletion of *SCS2*, and *scs2* mutants

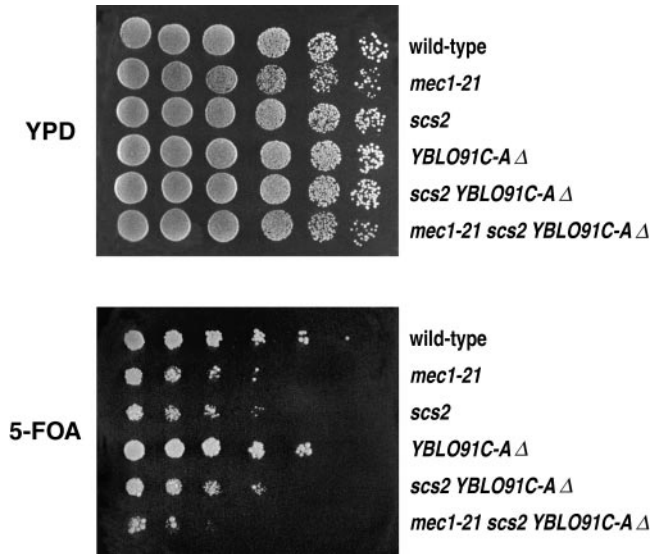


FIGURE 3.—*SCS2* is required for telomeric silencing. Both *SCS2* and a related ORF, *YBL091C-A*, were deleted and the resulting strains were assayed for telomeric silencing. The strains tested were RCY269-6a (wild type), RCY269-3d (*mec1-21*), RCY269-4a (*scs2*), RCY269-2b (*YBL091C-A*Δ), RCY269-13c (*scs2 YBL091C-A*Δ), and RCY269-1b (*mec1-21 scs2 YBL091C-A*Δ).

were not more sensitive than wild-type strains to ultraviolet light, hydroxyurea, or methyl methane-sulfonate (data not shown).

Silencing of the *HML* locus requires many of the same proteins necessary for telomeric silencing (APARICIO *et al.* 1991). Silencing at *HML* can be conveniently monitored using a strain in which the wild-type *TRP1* gene has been inserted at *HML* (STONE and PILLUS 1996). Silencing results in a tryptophan-requiring phenotype. As shown in Figure 4, the *scs2* mutation does not reduce silencing at the *HML* locus, although the *rap1-17* mutation, as expected (KYRION *et al.* 1993), does result in a silencing defect. We previously observed that *mec1-21* also reduced telomeric silencing without affecting silencing at *HML* (CRAVEN and PETES 2000).

*SCS2* shares 48% identity with an uncharacterized open reading frame YBL091C-A. This ORF lacks an ATG start site, but is transcribed (VELCULESCU *et al.* 1997). The strain RCY269-2b, which has a deletion of YBL091C-A, was viable and had wild-type levels of telomeric silencing (Figure 3). This deletion also has no effect on telomere length or sensitivity to HU (data not shown). Furthermore, a strain with a deletion of the YBL091C-A ORF and an *scs2* mutation has approximately the same telomeric silencing defect as the single *scs2*Δ mutant (Figure 3). We conclude that *SCS2* contributes to telomeric silencing, but that the related open reading frame YBL091C-A does not.

Double mutants of *mec1-21* and *tel1*Δ undergo loss of telomeric sequences and cellular senescence, followed by the emergence of a small number of surviving cells

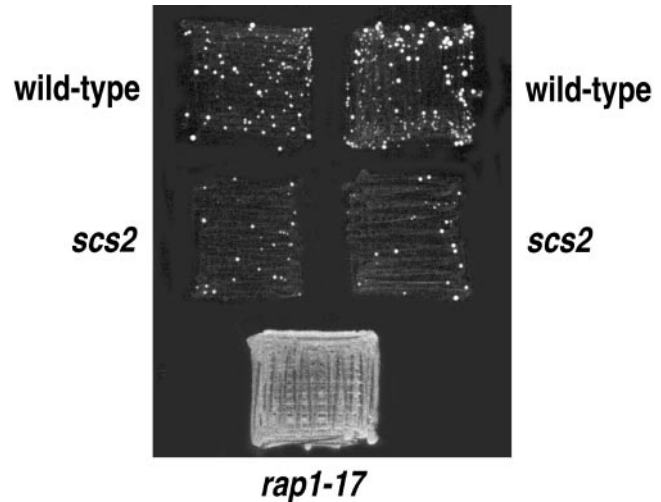


FIGURE 4.—*SCS2* is not required for mating-type silencing. Five strains were constructed containing the *TRP1* gene inserted at *HML*. The ability to silence *HML* results in poor growth on medium lacking tryptophan. Wild-type (RCY282-2a, left; RCY282-7c, right) and *scs2*Δ cells (RCY282-11c, left; RCY282-13d, right) were proficient for mating-type silencing, whereas *rap1-17* cells (RCY124-2a) were not.

(RITCHIE *et al.* 1999). A triple mutant *mec1-21 tel1*Δ *scs2*Δ strain underwent senescence at an accelerated rate compared to *mec1-21 tel1*Δ mutants (Figure 5, left side). In addition, the survivors derived from the triple mutant strain were less abundant and grew more slowly than *mec1-21 tel1*Δ survivors. We analyzed seven tetrads containing pairs of *mec1-21 tel1*Δ and *mec1-21 tel1*Δ *scs2*Δ spores. For each pair, the triple mutant senesced at an earlier stage of subculturing than the double mutant. At early stages of subculturing, telomere lengths in *mec1-21 tel1*Δ strains were the same as those in *mec1-21 tel1*Δ *scs2*Δ strains (data not shown), suggesting that the earlier senescence in the *mec1-21 tel1*Δ *scs2*Δ strains is not likely to reflect an effect on telomere length. The *mec1-21 scs2*Δ or *tel1*Δ *scs2*Δ mutants were viable and did not senesce even after extended subculturing (Figure 5, right side).

**The *scs2*Δ mutation is not suppressed by overexpression of *RNR1* or *INO1*:** The telomeric silencing defects of *mec1-21* and *dun1*Δ are suppressed by overexpression of the *RNR1* gene and by the *sml1* mutation (CRAVEN and PETES 2000); both of these alterations are likely to lead to elevated nucleotide pools (ZHAO *et al.* 1998). Neither overexpression of *RNR1* (Figure 6a) nor the *sml1* mutation (data not shown) reversed the telomeric silencing defect of *scs2*. These results suggest that *SCS2* and *MEC1* may affect different pathways required for telomeric silencing.

One model for the effect of the *scs2* mutation on telomeric silencing is that *scs2* cells have elevated levels of damage. In the presence of DNA damage, telomeric silencing proteins are recruited to the sites of the damage, resulting in loss of silencing; this recruitment re-



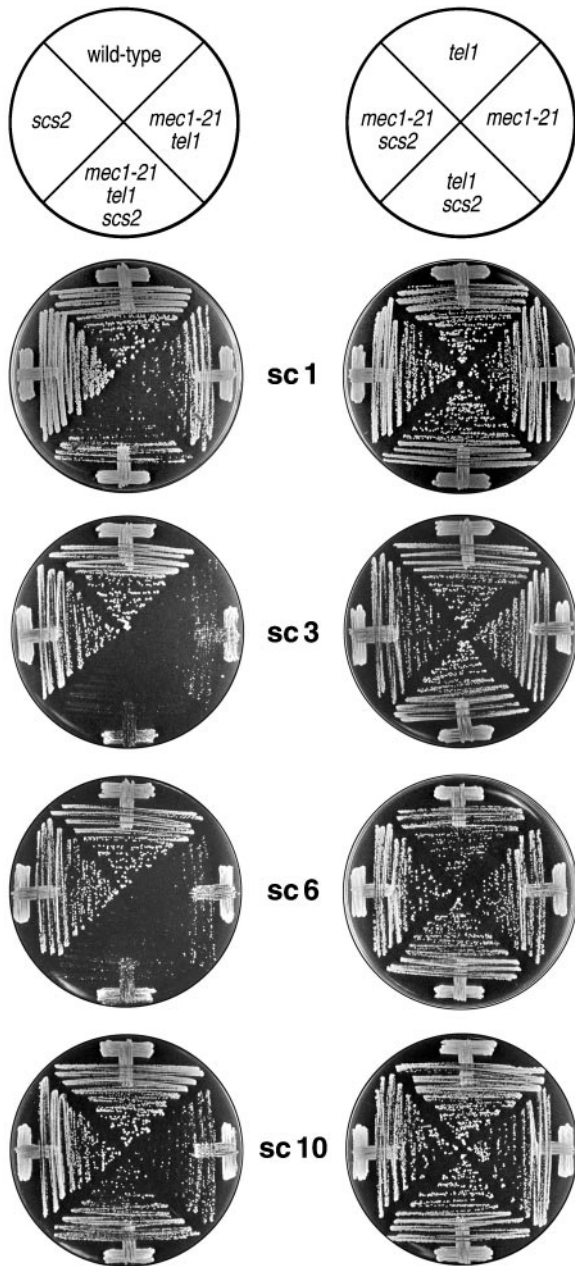


FIGURE 5.—The *scs2* mutation causes an increased rate of senescence in *mec1-21 tel1* cells. Strains derived from sporulating the diploid RCY305 were subcultured 10 times (sc1–sc10) on YPD-containing plates. The strain names were: RCY305-9d (wild type), RCY305-9c (*mec1-21 tel1*), RCY305-9b (*mec1-21 tel1 scs2*), RCY305-9a (*scs2*), RCY305-7a (*tel1*), RCY305-7b (*mec1-21*), RCY305-7c (*tel1 scs2*), and RCY305-7d (*mec1-21 scs2*). The triple mutant *mec1-21 tel1 scs2* reproducibly had a faster rate of senescence than the *mec1-21 tel1* double mutant.

quires the Rad9p (MILLS *et al.* 1999). Consequently, if the silencing defect in *scs2* strains reflects increased levels of DNA damage, strains with mutations in both *scs2* and *rad9* would have increased telomeric silencing. We examined telomeric silencing in isogenic *RAD5 TEL-XV<sub>L</sub>::URA3* strains with the following genotypes: *scs2* (RCY307-3c), *rad9* (RCY307-4a), and *scs2 rad9* (RCY307-

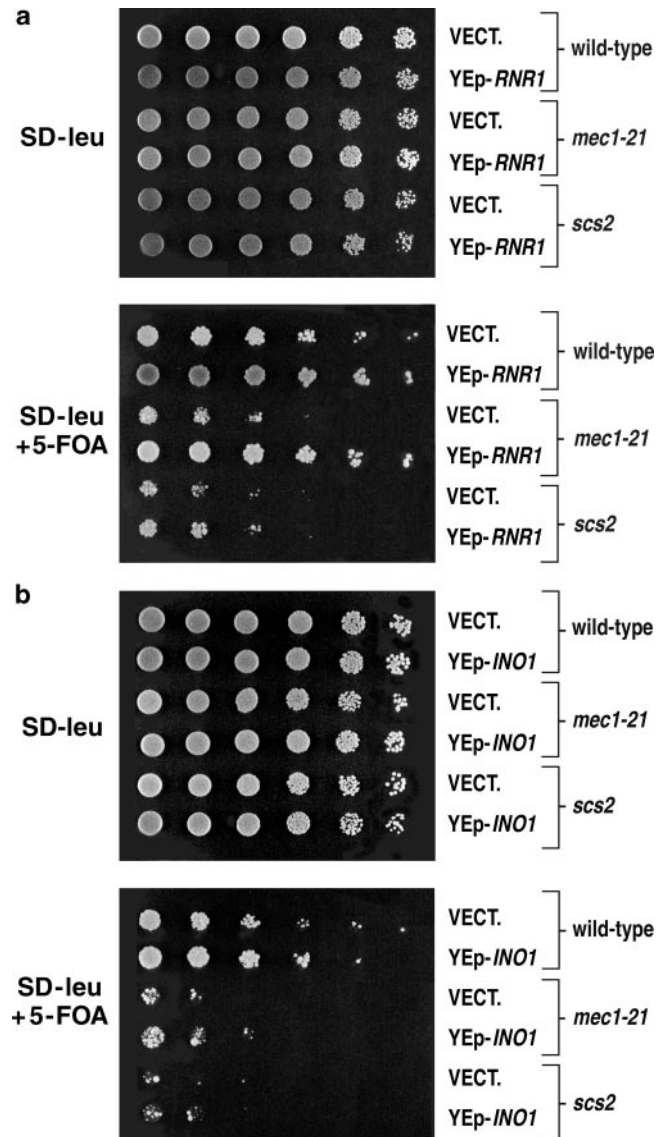


FIGURE 6.—The *scs2* telomeric silencing defect is not suppressed by *RNR1* or *INO1* overexpression. The silencing assay was the same as used in Figure 1. (a) The wild-type (RCY269-6a), *mec1-21* (RCY269-3d), and *scs2* (RCY269-4a) strains were transformed with the control vector YEplac181 (VECT.) or the overexpression plasmid pRC11 (YE-*RNR1*). (b) The same strains used in a were transformed with the *INO1* overexpression plasmid pJH318 (YE-*INO1*).

2c). The double mutant strains had the same silencing defect as the *scs2* single mutant strain (data not shown), ruling out the simplest forms of this model.

*SCS2* was originally identified as a suppressor of the inositol auxotrophy of *CSE1* (choline sensitive, a dominant mutation) and *ire15* (inositol requiring) mutants (KAGIWADA *et al.* 1998). Both of these mutants lack the ability to express the *INO1* gene, which encodes the enzyme inositol-1-phosphate synthase (DEAN and HENRY 1989). The Ino1p catalyzes the conversion of glucose-6-phosphate to inositol-1-phosphate, the first committed step in inositol phosphate synthesis. The *scs2Δ* mutants are

leaky inositol auxotrophs at elevated temperatures, and this auxotrophy is suppressed by overexpression of the *INO1* gene (KAGIWADA *et al.* 1998). Overexpression of *INO1*, however, did not suppress the telomeric silencing defects of *scs2Δ* or *mec1-21* cells (Figure 6b). In addition, deletion of the *INO1* gene did not affect telomeric silencing. We conclude that the effects of the *scs2* mutation on silencing are not mediated through *INO1*.

**The *scs2Δ* telomeric silencing defect is suppressed by overexpression of *SIR3* or by the *rif1* mutation:** One important component of telomeric silencing appears to be the level of Sir3p bound at and near the telomere. Sir3p binds to the carboxy terminus of the telomere-binding protein Rap1p in competition with Rif1p (MORRETTI *et al.* 1994; HECHT *et al.* 1996). Telomeric silencing is decreased by *sir3* mutations (APARICIO *et al.* 1991) and elevated by overexpression of Sir3p (RENAULD *et al.* 1993) or mutations of *RIF1* (KYRION *et al.* 1993). The *scs2Δ* telomeric silencing defect was completely suppressed by multiple copies of the *SIR3* gene (Figure 7a) and by the *rif1* mutation (Figure 7b). Overexpression of Sir3p also suppressed the telomeric silencing defect of *mec1-21* (Figure 7a).

One interpretation of the observation that the *scs2* telomeric silencing defect is suppressed by the *rif1* mutation is that Scs2p negatively regulates the function of Rif1p. As described above, Scs2p overexpression suppresses the inositol auxotrophy associated with mutations in the *INO1* pathway. To find out whether the *rif1* mutation might interact with mutations in the *INO1* pathway, we examined the ability of isogenic spores (derived from the diploid RCY346) of the wild-type, *ino1*, *rif1*, and *ino1 rif1* genotypes to grow on medium lacking inositol. Wild-type and *rif1* strains grew normally, whereas *ino1* and *ino1 rif1* strains grew very slowly (although at the same rates). Thus, Rif1p does not appear to affect the *INO1* pathway.

Both telomeric heterochromatin (GOTTA *et al.* 1996) and Scs2p (KAGIWADA *et al.* 1998) localize to the perinuclear region of the cell, raising the possibility that Scs2p might bind directly or indirectly to telomeres. To test this possibility, we tagged the Scs2p with an HA epitope (RCY309); we also constructed a strain (RCY310) containing HA-tagged Sir3p, a known telomere-binding protein (HECHT *et al.* 1996). The HA-tagged Scs2p protein was proficient for telomere silencing and could be readily detected by Western blot. Using formaldehyde crosslinking and chromatin immunoprecipitation (ChIP) analysis (details in MATERIALS AND METHODS), we failed to detect Scs2p bound to the telomere, although we could readily detect the binding of telomeric sequences to an HA-tagged version of the Sir3p control (data not shown). Thus, it is unlikely that Scs2p affects telomeric silencing through a stable direct interaction with telomeric heterochromatin. We cannot exclude the possibility of an unstable association of Scs2p with the telomere.

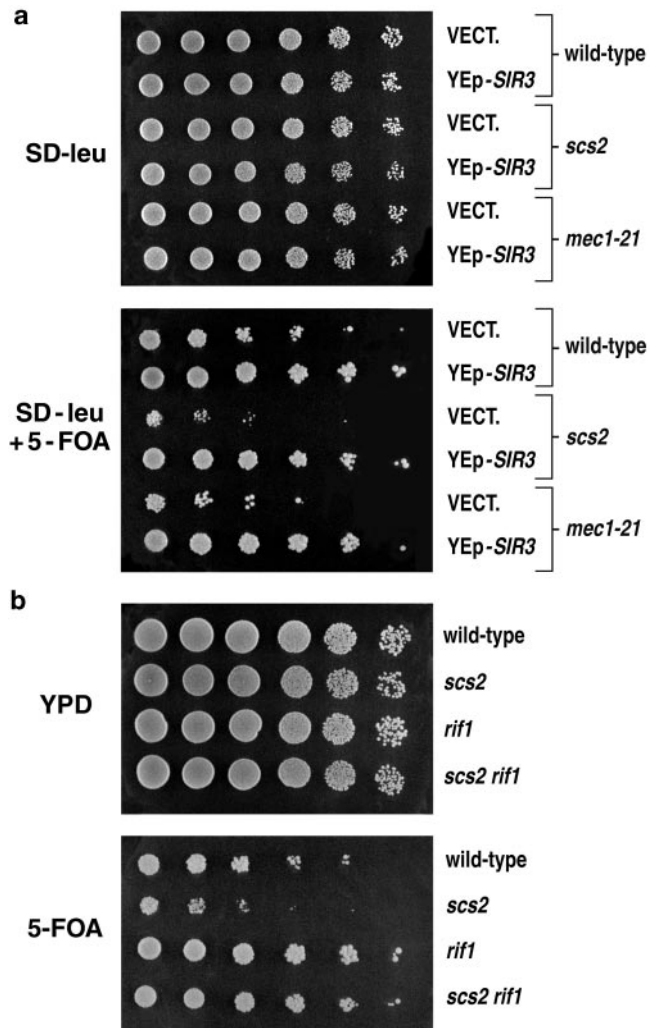


FIGURE 7.—The *scs2* telomeric silencing defect is suppressed by overexpression of Sir3p or by the *rif1* mutation. (a) Wild-type (RCY269-6a), *mec1-21* (RCY269-3d), and *scs2* (RCY269-4a) strains were transformed with the control vector YEplac181 (VECT.) or with the *SIR3* overexpression plasmid pLP304 (YEplac181-SIR3). Telomeric silencing assays were performed as described previously. (b) Telomeric silencing assays were done for wild-type (RCY280-4b), *scs2* (RCY280-6b), *rif1* (RCY280-1b), and *scs2 rif1* (RCY280-3b) strains.

## DISCUSSION

The major conclusions of this study are: (1) telomeric silencing defects caused by overexpression of Mec1p or by the *mec1-21* mutation are suppressed by overexpression of *SCS2*; (2) deletion of *SCS2* causes a partial loss of telomeric silencing and accelerates senescence in *mec1-21 tel1* cells; and (3) loss of silencing in *scs2Δ* cells is suppressed by multiple copies of *SIR3* and loss of *RIF1*, but not by multiple copies of *RNR1* or *INO1*. Multicopy suppressors function through one of three mechanisms: activation or increase in levels of a downstream target in the same pathway as the mutated protein, inactivation of an inhibitory pathway of the mutated protein, or activation of a parallel pathway of the mutated protein. We discuss our results in the context of these possibilities.



Previous studies identified *RAD53*, *DUN1*, and *RNR1* as multicopy suppressors of the essential function of Mec1p (SANCHEZ *et al.* 1996; DESANY *et al.* 1998). These proteins are thought to function as downstream effectors in the same DNA repair checkpoint pathway as Mec1p (reviewed by LOWNDES and MURGUIA 2000). Mec1p is a protein kinase (MALLORY and PETES 2000; PACIOTTI *et al.* 2000) and several of the proteins downstream of Mec1p, such as Rad53p (SANCHEZ *et al.* 1996), are phosphorylated in a Mec1p-dependent fashion *in vivo*.

Our observation that overexpression of Scs2p suppresses the telomeric silencing defects of *mec1-21* and *dun1* is consistent with the possibility that Mec1p and Scs2p act in the same pathway. There are, however, several observations that are difficult to explain by this hypothesis. First, the *scs2Δ* silencing defect is not suppressed by elevated nucleotide pools, a condition that suppresses the *mec1-21* silencing defect. Second, the *scs2Δ* mutation is suppressed by loss of the *RIF1* gene, which does not affect the *mec1-21* silencing defect (CRAVEN and PETES 2000). Third, it is unlikely that Scs2p is a direct substrate for the kinase activity of Mec1p because an epitope-tagged version of Scs2p does not exhibit altered expression, mobility, or processing in *mec1-21* cells (data not shown). Although none of these arguments are conclusive, the simplest interpretation of the data is that Scs2p does not function in the same pathway affecting telomeric silencing as Mec1p.

An alternative explanation is that the Scs2p affects a pathway that competes with that regulated by Mec1p. If Scs2p were part of a pathway that inhibits Mec1p and if Scs2p overexpression disrupted this pathway, then Scs2p overexpression might restore silencing to *mec1-21* mutants. This model, however, does not explain the loss of silencing observed in *scs2Δ* strains, since loss of Scs2p should result in more efficient Mec1p-mediated telomeric silencing.

Consequently, we favor a model in which Scs2p regulates telomeric silencing in a pathway operating independently of Mec1p. Since the silencing defect of *scs2Δ* mutants is restored by altering the balance of Sir3p at the telomere by overexpression of Sir3p or loss of the competing Rif1p (Figure 7), one possibility is that Scs2p acts in the Sir3p/Rif1p pathway of silencing. By this model, Scs2p could positively regulate Sir3p proteins or negatively regulate Rif1p. Scs2p is related to the *Aplysia californica* synaptobrevin/vesicle-associated membrane protein (VAMP)-associated protein VAP-33, which functions in protein secretion (SKEHEL *et al.* 1995; LAPIERRE *et al.* 1999). Scs2p is associated with the endoplasmic reticulum (ER), although *scs2* strains do not have defective protein secretion (KAGIWADA *et al.* 1998). KAGIWADA *et al.* (1998) suggested that Scs2p might be a membrane-bound transcription factor that is released from the ER to the nucleus in response to certain cellular signals. If the Scs2p is a transcriptional activator of one or more silencing proteins, then overexpression of

Scs2p might relieve the telomeric silencing defect caused by overexpression or mutation of the Mec1p. Loss of Scs2p might result in a diminished level of silencing proteins and partial loss of telomeric silencing. Although we observed no effect of the *scs2* mutation on the silent mating-type loci, telomeric silencing is often more sensitive to subtle changes in the levels of silencing proteins than silencing at the mating-type loci (APARICIO *et al.* 1991). It is unlikely that Scs2p acts as a negative regulator of Rif1p, since strains with *rif1* mutations have elongated telomeres (HARDY *et al.* 1992) and *scs2* strains have wild-type-length telomeres. Thus, for Scs2p to be a negative regulator of Rif1p, the telomere-length regulatory activity of Rif1p would have to be separable from its effects on telomeric silencing.

One alternative intriguing possibility is that Scs2p alters silencing indirectly through the synthesis or processing of phospholipids. These lipids might serve as docking sites for heterochromatin on the nuclear membrane or be part of a signaling cascade that regulates silencing. One argument against this model is that the only known target of Scs2p in the phospholipid pathway, the *INO1* gene, has no effect on silencing when deleted or overexpressed.

In summary, *SCS2* is involved in regulating telomeric silencing. Although we identified *SCS2* in a genetic screen for genes that were multicopy suppressors of a silencing defect associated with Mec1p overexpression, our results suggest that Scs2p regulates telomeric silencing in a different pathway from Mec1p.

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